Ecotoxicity Assessment of Weathered Waste Oil in a Mexican Wetland

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Abstract- **The present study applied the ecotoxicity assessment through of a battery of bioassays used to determine toxicity potential in a tropical wetland impacted for oil waste industry. The bioassays applied were Microtox®, Eisenia foetida, Glycine max and Triticum aestivum. The total petroleum hydrocarbons (TPH) in soil was varied to 200,000 mg kg-1. The PAHs between compounds detected was the benzo(a)pyrene (B(a)P). The results of bioassays showed a higher potential toxicity obtained in the zone 2 and 4 respect to control zone (p<0.001). The results from the MANOVA analysis, determined the zone 2 with the bigger ecotoxicity potential regarding to others zones. The results showed a gradient of sensitivity soil toxicity as follows: Microtox® > Eisenia foetida > Glycine max > Triticum aestivum (p < 0.05). In case of both values, LC50 and EC 50 for (B(a)P) the best relationship (r ≈ 0.9, P<0.05) was obtained with phytoassays with G. max and T. aestivum.**

Keywords*- Ecotoxicity; TPH; PAH; B(a)P; Oil Waste; Bioassays; Wetland; Phytoassays; Tropical Wetland*

I. INTRODUCTION

Wetlands are critically important wildlife habitats, often serving as breeding grounds for a wide variety of animal life. Wetlands recharge groundwater supplies and moderate stream flow by providing water to streams. Wetland vegetation and microorganisms also use excess nutrients for growth that can otherwise pollute surface water, such as nitrogen and phosphorus from fertilizers (US EPA, 2006).

Pollution, especially near urban areas or industrial, remains a serious threat to ecosystems. Fortunately, some countries have enacted special laws to protect wetlands, but much diligence is needed to that these protective measures are actively enforced. Since there is an absence of regulatory framework in Mexico to set toxicity test in order to promote environmental protection, remediation or ecological restoration, we used a set of bioassays to assess Mexican wetland which has been impacted by weathered waste oil.

Bioassays provide important information for the assessment of pollutant effects of chemicals or environmental samples. In contrast to chemical analyses, they also detect effects of multiple contaminants and metabolites (Eisentraeger et al., 2005). The use of a set of tests on species at different levels of biological organization and of biological approaches to complement physico-chemical analyses has been recommended for a refined evaluation of environmental risk.

Terrestrial plant and invertebrate (earthworm, collembola) tests have been selected on the basis of their ability to measure chemical toxicity to ecologically relevant test species during chronic assays which include at least one reproductive component among the measured endpoints (Eom et al., 2007).

Between pollutants of concern, we can identify total petroleum hydrocarbons (TPH), including polycyclic aromatic hydrocarbons (PAH). These compounds are residues from combustion, coke production, petroleum refining, and other high-temperature industrial processes (Bispo et al., 1999).

II. MATERIALS AND METHODS

A. Methods for Soil Sampling

Soil samples contaminated with hydrocarbons were taken from a tropical wetland (total area = 72 km^2) located in the east central part of Mexico, between 17°10' and 22°38' N and between 93°55' and 98°38' W. Samples were taken from the top 50 cm soil layer (US EPA, 1998), as hydrophobic compounds are usually adsorbed (Riser-Roberts, 1998). Due to the presence of petroleum industry, the study area received irregular and uncontrolled disposal of waste oil for more than 5 decades. The study area was divided in four contaminated zones (Z) and one zone considered as control zone (Figure 1), according to previous studies that reported different levels of contamination (Uribe-Hernández et al, 2004), having the control zone the lowest levels of total petroleum hydrocarbons (TPH) and none of the 16 EPA priority polyaromatic hydrocarbons (PAH). A total of 30 samples were taken per zone. The results show the mean value of triplicate samples.

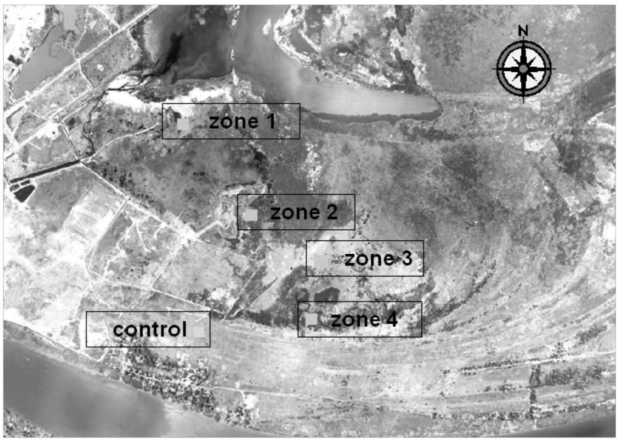


Figure 1 Wetland in Veracruz, Mexico. The aerial photo, scale: 1:5,000, shows the four zones of the study area. Between 17°10’ and 22°38’ North and between 93°55’ and 98°38’ West. 17°10’and 22°38’ North

B. Methods For Hydrocarbon Analyses

Soil extract was obtained as follows: 50 g of soil was placed into an Erlenmeyer flask in addition to 1 g of anhydrous sodium sulphate (Sigma Aldrich®, Houston, TX, USA) and 50 ml dichloromethane (Merck, Bedford, MA, USA), stirring for 30 min, leaving it to rest for approximately

45 min, and filtering through fiberglass (Coatings Inc., Buffalo, NY, USA) to obtain the pure extract, finally covering hermetically to avoid evaporation of the solvent.

1) Total Petroleum Hydrocarbons:

Duplicate samples (40 g wet wt) of oily soils from each treatment were taken on each zone for determinations of TPH. Methylene chloride solvent was used in the Soxhlet extraction. The solvent extract was treated with silica gel to remove polar compounds and analysed by an infrared (IR) analyser (Perkin-Elmer) according to EPA Method 418.1 as TPH-IR. The calibration standard used in the TPH-IR method was 25% (v/v) n-hexadecane, 37.5% (v/v) isooctane, and 37.5% (v/v) chlorobenzene; absorption was measured in the IR spectral range of 3400–3500 cm-1 (USEPA, 1979). TPH analysis was performed using the EPA 418.1 method, with a detection limit (dry base) of 68.8 mg kg-1. This method was used only for hydrocarbon contamination screening.

2) Polycyclic Aromatic Hydrocarbons:

Polycyclic compounds (two-, three-, and, four-, and five-ring PAH) were extracted using sonication and methylene chloride from 2 g of soil according to EPA Method 3550 and analysed by a direct injection GC/MS determination based on EPA Method 8270 (USEPA, 1988).The detection limit for each hydrocarbon is listed in Table 1.

TABLE I AVERAGE TPH AND PAH MEASURED IN THE SOIL OF THE WETLAND

Zone	TPH (mg kg^{-1})	PAH (mg kg^{-1})				
		Phenanthrene	Anthracene	Fluorene	Benzo(a) Anthracene	B(a)P
Z1	10,904 ±1,312	6.32 ±1.02	1.51 ±0.11	DL^{c}	4.1 ±0.24	1.22 ±0.12
Z2	780,000 ±63,000	37.71 ±9.38	$<DL^{b}$	9.03 ±1.23	DL^{d}	2.03 ±0.22
Z3	298,000 ±28, 022	$<DL^{a}$	3.92 ±0.98	DL^{c}	DL^{d}	1.29 ±0.23
Z4	19,117 ±1,107	2.63 ±0.23	1.08 ±0.97	DL^{c}	DL^{d}	2.89 ±0.11
Control	105 ± 11	$<DL^{a}$	$<DL^{b}$	DL^{c}	DL^{d}	DL^{e}

DL= Detection limit (mg kg^{-1}) ^{a}0.002, ^{b}0.007, ^{c}0.065, ^{d}0.067, ^{e}0.06.

C. Bioassays

To determine toxicity potential, bioassays were carried out at two levels of response; the first instance consisted of calculating the lethal toxicity (LC_{50}). In the second approach, we determined the sublethal toxic effects to obtain the effective concentration (EC_{50} and EC_1), with the latter used to determine clean up levels.

The bioassays for toxicity were selected taking into account the ecologic niche for biologic group. *Glycine max* in addition to *Triticum aestivum* as plants in phytoassay analysis, *Eisenia foetida* as a mesofauna representative species, and Microtox® as microbiological bioassay. All tests were carried out in triplicate and results are the mean value.

Germination tests are important for soil evaluation because the toxic effects are observed by the inhibition or promotion of the seed and root, and, at later times, the growth process. For germination, certified seeds provided by the Productora Nacional de Semilla [National Producer of Seeds], Mexico, were used. Test conditions followed the guidelines of OECD (1984) and ISO (2003b) for *T. aestivum* and *G. max* in triplicate. Test temperature was kept at 22 ± 1 °C, exposure time was 5 days, and the test volume was 15 ml (Petri dish diameter, 40 mm), using a light cycle of 16 h light/8 h dark (light 9000 lx). When 65% of the seeds from the negative test were germinated, the number of germinated seeds in each TPH extracts dilutions (0, 3.125, 6.25, 12.5, 25, 50, and 100%), were counted if their root was > 5 mm. 2-chloroacetamide (35 mg/l) was used as a positive control and distilled water as a negative control. The germination test was maintained within an environmental test chamber at a controlled temperature (23/15 °C day/night) and a 16:8 h (light: dark) photoperiod. A total of 10 seeds were exposed per dish. Dose-response curves were obtained to determine the lethal concentration 50 (LC_{50}) of the toxicant at different concentrations.

The phytotoxicity test to evaluate *Triticum aestivum* and *Glycine max* length was performed as follows. After the germination test, we transplanted each plant into agrolita (10 g) containing the TPH extracts (0, 3.125, 6.25, 12.5, 25, 50, and 100% from soil extract). The 16:8 h light: dark photoperiod was maintained, using cultivation lamps Philips SON/T Plus (400 W). All replicates were randomized for the position that they occupied. Testing time lasted 21 and 14 days for *Glycine max* and *Triticum aestivum*, respectively, to leave enough time for the first foliar primordia growth (US EPA, 1996). After the TPH exposure period had ended, biomass, in addition to the stem and root length, was measured in triplicate. The half maximal effective concentration (EC_{50}) was calculated for TPH, and the dose-response curve was obtained. In order to calculate the effective concentration (EC_1 and EC_{50}) for B(a)P for both *G. max* and *T. aestivum*, a dose-response curve was calculated using the dilutions mentioned above.

For the acute toxicity test (96 h) using *E. foetida*, organisms were acclimated before the bioassay took place. The lab conditions were established according to the ISO 11268 method (ISO, 2003a). The test was performed using the dilution extracts at 0, 3.125, 6.25, 12.5, 25, 50, and 100% with three replicates for each treatment. Placing Whatman cellulose paper disks No. 1 inside a Petri dish (9-cm diameter), 1 ml of the soil extract was distributed homogeneously on the paper, evaporating the solvent during 10 min. Then, 1 ml distilled water and 0.5 ml dimethyl sulfoxide at 1% were added as a vehicle for the assimilation of the toxicant. For the positive (+) and negative (-) tests, 1 ml dichloromethane and distilled water were added, respectively. A total of 10 organisms were exposed per dish. Dose-response curves were obtained to determine the lethal concentration 50 (LC_{50}) of the toxicant at different concentrations.

Microtox® was the method used for the bacterial toxicity evaluation.). The bioluminescence reduction on *Vibrio fischeri* was measured with a photomultiplier coupled to a light sensor and toxicity results are expressed as EC_{50} at a given time (Anzur, 1998). Soil toxicity was determined by analyzing elutriates from the contaminated soils, which were obtained with 1 g soil in 5 ml of a 35% NaCl solution, and making an extraction (at 25°C) by ultrasound (Cole-Parmer mod 8845-40, Vernon Hills, IL, USA) for 15 min (EPA 3552C method) (US EPA, 2000). The sample was filtered using membrane filters of 45-µm pore, and toxicity was analyzed during 5 and 15 min, by measuring the light emitted by bacteria, in comparison with a negative control. Toxicity was expressed as “Toxic Units, TU”, where TU = $100/EC_{50}$.

D. Statistical Analysis

Probit regression analysis was used to fit and determine the dose-response curve to calculate the lethal concentration

50 (LC_{50}) and effective concentrations 1 and 50 (EC_1 and EC_{50}, respectively). For each bioassay, one-way ANOVA was calculated to determine if there were statistically significant differences in the observed responses among the four zones and the control zone and to analyze if the answer was related to the degree of contamination in each area. MANOVA were performed for all the bioassays and their endpoints, together with the hydrocarbon chemical analyses, performing multiple post hoc tests by Tukey Honestly Significant Difference test. All statistical analyses were performed using the software SPSS® V.10.

III. RESULTS AND DISCUSSION

The results of the TPH and PAH levels are shown on Table 1. The highest level of TPH was found in Z2, followed by Z3; and Z1 and Z4 were relatively similar with the lowest levels. Although the control sample was not completely free of hydrocarbons, it had the lowest TPH level (105 mg kg^{-1}) and it had none of the 16 priority PAH. The rest of the priority PAH, not included on Table 1, were not detected in any of the zones or the control one.

In an average of 622 soil samples, TPH fractions contained 21.75 ± 16.12% saturated hydrocarbons, 32 ± 23% aromatic hydrocarbons, and 46.25 ± 28.28% asphaltenes. From the 16 EPA priority PAH analyzed, benzo(a)pyrene (B(a)P) exceeded the maximum permissible limit (2 mg kg^{-1}) in soil according to the Mexican Regulation (DOF, 2005). This difference in values could be an important reason for the big difference in bioassay responses (both lethal and sublethal) among the different zones, as will be described below.

In the case of the lethal test with soybean (*Glycine max*), the highest lethality (42%) was obtained in Z2, with significant differences ($p < 0.05$) as compared to control. Samples that showed the lowest germination (50%) came from Z2 as well. The Z2 was the most toxic due to the high TPH levels (LC_{50} = 17.8 mg kg^{-1}), followed by Z4 and Z1 (Table 2). The toxicological gradient was as follows: Z2 > Z4 > Z1 > Z3.

TABLE II INHIBITION OF GERMINATION (%) AND LC_{50} FOR *G. MAX* AND *T. AESTIVUM*

Zone	Maximal inhibition of *G. max* germination (%)	Maximal inhibition of *T. aestivum* germination (%)	LC_{50} for *G. max* (mg kg^{-1} TPH)	LC_{50} for *T. aestivum* (mg kg^{-1} TPH)
Z1	39	31	518.3*	44.51
Z2	77*	63*	17.8*	3.56
Z3	38	30	NE	2072.12*
Z4	36	62	66.5*	7.48
Control	3	5	NA	NA

NE =No biological effect was observed in samples from Z3.

*Significant difference ($p < 0.005$). NA=Not applicable.

T. aestivum germination showed a similar pattern to that of *G. max* (Table 2). The highest toxicity was registered in Z2 with an LC_{50} = 3.56 mg kg^{-1} TPH. The gradient of toxic potential was also consistent with the results obtained with soybean. Results of *T. aestivum* germination using the dilution of soil extracts 0, 3.125, (6.25, 12.5, 25, 50, and 100%) showed the same significant difference ($p < 0.001$) between the control and the samples from zones. A dose-response curve using the above dilutions was performed, from which the inhibition of germination was obtained. Table 2 shows the maximal inhibition of germination and LC_{50} for both plants.

In addition to the germination test, stem and root length (Table 3) was registered as an indirect measurement of the plant growth (Gomot-De Vaufleury, 2000). Considering this growth, there was significant difference ($p < 0.05$) between control and Z1 and significant difference ($p = 0.0017$) among control, Z2, and Z4.

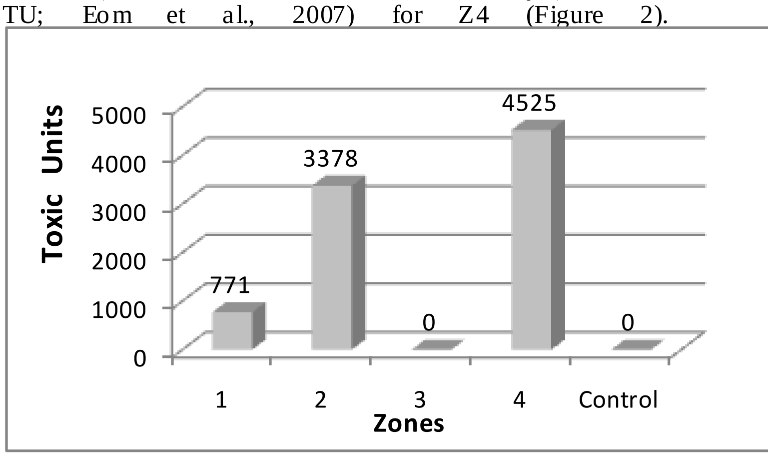
TABLE III STEM AND ROOT LENGTH IN ADDITION TO PLANT BIOMASS FOR G. MAX AND T. AESTIVUM

Zone	Stem length (cm) for G. max	Stem length (cm) for T. aestivum	Root length(cm) for G. max	Root length (cm) for T. aestivum	Biomass G. max	Biomass T. aestivum
Z1	6.1	9.7	2.6	16.5	0.36	0.09
Z2	6.2	7.8	4.1	2.6	0.43	0.04
Z3	5.0	6.7	5.4	11.8	0.74	0.09
Z4	8.5	6.8	3.9	12.5	0.58	0.08
Control	16.4	12.7	6.5	16.6	1.05	0.12

An important aspect to deal with when assessing different toxicant's answers is the different sensitivity associated with the type of toxicant, which implies also different mechanisms of toxicity. Such is the case of germination inhibition by both TPH and PAH, their phytotoxicity is frequently associated with a blockade of seed imbibitions, referred as narcosis, which causes a lack of root emergency in the seed (Besalatpour et al., 2008). However, both root length and stem growth have more to do with PAH than with TPH, possibly due to endocrine disruption caused by these aromatic compounds.

Results obtained at the first level of response (lethal) using *Eisenia foetida* showed statistical differences among zones ($p < 0.05$). At the second level of response (sublethal), Z2 showed an elevated mortality (83%), whereas in Z1, 50% of the organisms died. In the rest of the organisms, only a decrease in mobility was observed. In Z3 and Z4, the highest mortality of *E. foetida* (40%) occurred during the bioassay using the soil extract from Z3. In Z4, 10% mortality was measured. Using the dose-response curve to obtain LC_{50} for *E. foetida*, the highest toxicity was observed in Z2, with an LC_{50}= 310 mg kg^{-1} TPH. The toxicological gradient was as follows: Z2 > Z1 > Z3 > Z4. In Z2, the EC_1 = 0.047 mg kg^{-1} and 0.17 mg kg^{-1} in Z4.

The toxicity values obtained with Microtox® were the highest in Z4, followed by Z2 and Z1. Toxic Units (TU= $100/EC_{50}$) exceed the Microtox scale for toxicity (over 4500 TU; Eom et al., 2007) for Z4 (Figure 2).



0= Not detected

Figure 2 Mean Toxic units at 15 min obtained with Microtox®

Using the results from the sublethal response, the threshold level was calculated. This level, which is equivalent to EC_1, represents the lowest dose of a chemical at which a specified measurable effect for wetland biota is observed and, below it, no effect can be observed.

Sublethal results of plant length and biomass of *G. max* and *Triticum aestivum* using soil extract and Microtox® results suggest consistently that Z4 is the zone with the greatest relation in the dose-response curve (Table 4).

TABLE IV TPH EFFECTIVE CONCENTRATION (MG KG^{-1}) FOR PLANTS AND MICROTOX®

Zone	Equation	EC_1	EC_{50}
	Glycine max		
Z4-SL	y = -1.8229x + 16.755, r = 0.94	12600.2	15901.5
Z4- RL	y = -0.4869x + 7.1526, r = 0.88	5450.3	10359.2
Z4-B	y = -0.0761x + 0.9897, r = 0.69	843.9	5922.2
	Triticum aestivum		
Z2-SL	y = -1.496x + 15.029, r = 0.78	33515.4	42759.0
Z2-RL	y = -1.755x + 20.482, r = 0.64	45623.1	55628.2
Z2-B	y = -0.0122x + 0.177, r=0.60	2.6	11370.9
	Microtox ®		
Z2	Logy= 0.8583xLog +1.526, r=0.93	1.6	3.0
Z4	Logy= 0.7239xLog +2.183, r=0.73	2.1	126.6

SL = Stem length, RL = Root length, B = Biomass.

When performing the MANOVA test (Table 5) using as dependent variables the LC_{50}, EC_{50} and EC_1, and as variation factors the study areas and toxicity bioassays, we observed the following gradient of toxicity for the studied areas: Z2> Z4> Z3> Z1> ZC, being the control zone the least toxic ($F_{4, 20}$ = 20.1, p <0.001). This battery of tests and evaluated responses confirmed Z2 with the highest toxic potential in terms of both PAH and TPH and for both lethal (LC_{50} $F_{4, 40}$ = 2.7, p = 0.01) and sublethal responses (EC_1 = 16.89 with $F_{4.40}$, p = 0.004).

TABLE V MANOVA RESULTS

Source	Dependent Variable	df	Mean Square	F	Significance
Model	LC_{50} [a]	8	2027.892	2.827	.017
	EC_1root [b]	8	398901870.301	4.988	.000
	EC_1biomass [c]	8	1790938177.457	11.802	.000
	EC_1stem [d]	8	425806569.171	20.195	.000
Zone	LC_{50}	4	1936.769	2.700	.048
	EC_1root	4	192438345.532	2.406	.070
	EC_1biomass	4	719808552.139	4.744	.004
	EC_1stem	4	356156540.974	16.892	.000
Bioassay	LC_{50}	3	985.777	1.374	.268
	EC_1root	3	572695666.268	7.161	.001
	EC_1biomass	3	1596897022.264	10.524	.000
	EC_1stem	3	187060331.287	8.872	.000
Error	LC_{50}	32	717.221		
	EC_1root	32	79969080.559		
	EC_1biomass	32	151742550.271		
	EC_1stem	32	21084363.062		
Total	LC_{50}	40			
	EC_1root	40			
	EC_1biomass	40			
	EC_1stem	40			

[a] r^2= 0.41 (adjusted r^2= 0.26), [b] r^2= 0.55 (adjusted r^2=0 .44), [c] r^2= 0.74 (adjusted r^2=0.68), [d] r^2=0.83 (adjusted r^2= 0.79)

Regarding the sensitivity of the bioassay, Microtox® test is more sensitive, followed by *E. foetida*, *G. max*, and finally the least sensitive was *T. aestivum* (p <0.001).

Bioassays using organisms with different ecologic niche by biologic group have proven to be efficient tools for the detection of acute and subchronic toxicity and bioavailability, although they do not evaluate the medium and long term effects. However, in this case, bioassays with different exposure ways were used to determine toxicity potential because they all have different sensitivity to soil toxicity. The difference in sensitivity to hydrocarbons (TPH and PAH) is determined mainly by two factors, firstly the type of pollutant and the type of organism, based on bioconcentration, biotransformation, and the extent of damage, and secondly the ability to recover from it.

Thus, the toxicity levels would be a function of the soil composition of wetland, because all these organisms have a close interaction with the soil as substrate for their lifestyle. *V. fisheri* due to the direct exchange of gases and materials through the bacterial cell membrane, *E. foetida* through the soft skin of the digestive tract, and stem and root length of *G. max* and *T. aestivium* (Hubalék et al., 2007).

Using the results from the MANOVA analysis, and taking into account all of the bioassays and their endpoints, together with the hydrocarbon chemical analyses, we arrived at decisions regarding the toxicity potential depending on ecologic niche by biologic group.

Despite high sensitivity, results for microbial trophic level (Microtox®) and primary consumers (*E. foetida*) were not significant (p <0.05) to determine toxicity potential. However, for primary producers level, there were significant differences, therefore considering the bioassays with wheat (*T. aestivum*), with higher sensitive, the level of toxic potential was 3,081.68 mg kg^{-1} TPH (p = 0.013), whereas in the case of soybean (*G. max*), the determined value was 1,376.58 mg kg^{-1} TPH (p <0.001).

From the 16 EPA priority PAH analyzed, B(a)P was the only that exceeded the maximum permissible limit according to the Mexican Regulation. Based on the importance of plants as primary producers, they were selected to conduct the assessment of soil (sublethal concentrations), as done by some authors (e.g., Baird et al., 2007; Wilkea et al., 2008). We performed interpolation of the EC_1 to use this concentration as endpoint of lower toxicity response and therefore of higher sensible response. Results are shown in Table 6.

TABLE VI BENZO(A)PYRENE EFFECTIVE CONCENTRATION (MG KG^{-1}) FOR *G. MAX* AND *T. AESTIVUM*

	***Glycine max* Zone 4**		***Triticum aestivum* Zone 2**	
	EC_1	EC_{50}	EC_1	EC_{50}
Stem	4.9092	12.9615	0.1029	6.3382
Root	1.1945	2.8898	0.3366	2.2587
Biomass		51.9643		21.2678

According to the results of root length with soybean using B(a)P (Table 6), the greatest toxic potential was found in Z4

with an EC_{50} = 2.8898 mg kg^{-1}, whereas that for wheat was found in Z2 with an EC_{50} = 2.2587 mg kg^{-1}, being wheat the most sensitive species. The B(a)P threshold value for soybean using root length was EC_1= 1.19 mg kg^{-1} whereas for wheat it was EC_1= 0.34 mg kg^{-1}. The minimal sensitive level obtained (0.34 mg kg^{-1}) is much lower than 3 mg kg^{-1} (ATSDR, 2010; Bradley et al., 1994), and 2 mg kg^{-1} as established in Mexico for agriculture use (DOF, 2005).

IV. CONCLUSIONS

Currently, only a few soil guidelines for TPH are available to protect terrestrially ecological receptors (US EPA, 1997). An absence of basic ecotoxicological datum restrains the development of ecological soil screening values for TPH (Wang, 2010). Therefore the use of bioassays is recommended for ecotoxicological evaluations to determine sensitive between bioassays and toxicity gradient between zones.

In addition to the hydrocarbon concentration levels, the ecologically relevant criteria for estimating the impacts of petroleum hydrocarbons are also important end points for risk assessment, which contributes to protect the biodiversity of the ecosystem.

ACKNOWLEDGEMENTS

Financial and material support for this study was provided by PEMEX and Instituto Mexicano del Petróleo. R. Uribe-Hernández and M. A. Amezcua-Allieri are grateful for SNI fellowship.

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